The Shoot-Specific Expression of γ-Glutamylcysteine Synthetase Directs the Long-Distance Transport of Thiol-Peptides to Roots Conferring Tolerance to Mercury and Arsenic[1]

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Thiol-peptides synthesized as intermediates in phytochelatin (PC) biosynthesis confer cellular tolerance to toxic elements like arsenic, mercury, and cadmium, but little is known about their long-distance transport between plant organs. A modified bacterial *γ-glutamylcysteine synthetase* (*ECS*) gene, *S1pt::ECS*, was expressed in the shoots of the *ECS*-deficient, heavy-metal-sensitive *cad2-1* mutant of Arabidopsis (*Arabidopsis thaliana*). *S1pt::ECS* directed strong ECS protein expression in the shoots, but no ECS was detected in the roots of transgenic plant lines. The *S1pt::ECS* gene restored full mercury tolerance and partial cadmium tolerance to the mutant and enhanced arsenate tolerance significantly beyond wild-type levels. After arsenic treatment, the root concentrations of γ-glutamylcysteine (EC), PC_2, and PC_3 peptides in a *S1pt::ECS*-complemented *cad2-1* line increased 6- to 100-fold over the mutant levels and were equivalent to wild-type concentrations. The shoot and root levels of glutathione were 2- to 5-fold above those in wild-type plants, with or without treatment with toxicants. Thus, EC and perhaps glutathione are efficiently transported from shoots to roots. The possibility that EC or other PC pathway intermediates may act as carriers for the long-distance phloem transport and subsequent redistribution of thiol-reactive toxins and nutrients in plants is discussed.

The long-distance transport of thiol-peptides containing γ-glutamylcysteine (EC) may play important roles in the distribution and processing of nutrients and toxins. Phytochelatins (PCs) and the low-M_r peptide intermediates in PC biosynthesis, EC, and glutathione (GSH) can chelate or covalently bond to various elemental nutrients and toxins and are required for the transport of many toxins into the vacuole. Besides being rich sources of nitrogen and sulfur, these peptides might be expected to act as carriers for the long-distance phloem transport of small molecules between shoots and roots. This latter supposition is supported by data showing that, when plants are iron starved, the levels of nicotianamine, an endogenous chelator of iron, are modulated in leaves and roots, and believed to enhance both xylem and phloem mobility and redistribution of nicotianamine-chelated iron (Inoue et al., 2003). A similar role can be proposed for the EC-containing peptides as long-distance transport and redistribution carriers of thiol-reactive nutrients, such as zinc and copper, and toxicants, such as arsenic, mercury, and cadmium. Supporting this hypothesis indirectly are data showing that the root-specific expression of Arabidopsis (*Arabidopsis thaliana*) PC synthase (PCS) in Arabidopsis resulted in increased levels of PCs and cadmium in shoots, presumably via long-distance xylem transport (Gong et al., 2003). In this article, we examine the long-distance movement of thiol-peptides from shoots down to roots by expressing a bacterial ECS protein in the shoots of an Arabidopsis ECS-deficient mutant using a shoot-specific, light-induced regulatory cassette.

The EC-containing peptides, GSH and PCs, play important roles in detoxifying thiol-reactive metals, such as cadmium and mercury, and the metalloid arsenic (Cobbett, 2000). The PCs are a family of peptides derived from EC and GSH, as shown in Figure 1 (Cobbett and Goldsbrough, 2002). The first enzyme in the PC biosynthetic pathway, EC synthetase (ECS), catalyzes the ATP-dependent formation of the unusual peptide bond between the γ-carboxyl group of Glu and the α-amino group of Cys to make EC (Jez et al., 2004). This reaction is believed to be the rate-limiting and committed step in PC biosynthesis (Fig. 1; Noctor

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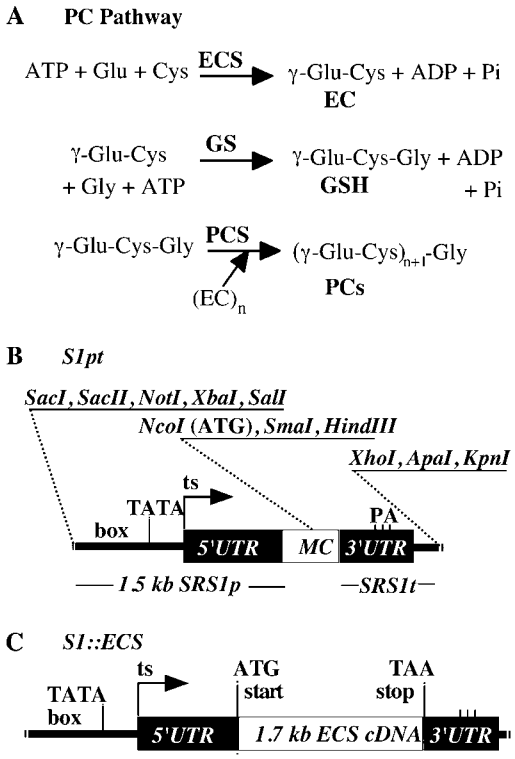

Figure 1. Enzymatic steps and transgenes affecting PC biosynthesis. A, Three enzymes are required to synthesize PCs from common amino acids. B, Map of *S1pt*, the light-induced, shoot-specific expression cassette containing the promoter, 5′-UTR, multicloning site, 3′-UTR, and terminator region with polyadenylation sequences from the soybean Rubisco small subunit gene *SRS1*. C, Physical map of the *S1pt*::*ECS* gene (*ECS* gene cloned into *S1pt*). Abbreviations: TATA box, the characterized sequence specifying the start of transcription; ts, start of transcription; PA, characterized poly(A) addition sites; ATG and TAA, initiation and termination codons; UTR, transcribed but untranslated regions; and MC, multicloning site.

et al., 1998; Zhu et al., 1999b). GSH synthetase (GS) catalyzes a second ATP-dependent step combining EC with Gly to make GSH (Jez and Cahoon, 2004). The role of GS in making PCs is complicated by potential feedback inhibition of ECS enzyme activity by GSH in some organisms (Richman and Meister, 1975), but the role of GS is unclear in organisms like plants, where PC synthesis itself can remove excess GSH. Thus, the functional activity levels of both ECS and perhaps GS may limit the production of GSH (Zhu et al., 1999a). PCs are synthesized in a third and reiterative enzymatic step in the pathway catalyzed by PCS (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999).

Considerable attention has been given to the roles of these thiol-peptides in the detoxification and accumulation of thiol-reactive elements like cadmium, mercury, and arsenic (Noctor et al., 1998; Zhu et al., 1999a, 1999b; Xiang et al., 2001; Li et al., 2004, 2005), based on an underlying assumption that the increased expression of these peptides might result in both tolerance and hyperaccumulation (Cobbett and Goldsbrough, 2002). The practical goal of this research has been to use these thiol-peptides in engineering the phytoremediation of soil and water contaminated with thiol-reactive elements (Meagher and Heaton, 2005; Meagher et al., 2006). Molecular genetic evidence that this pathway is essential to toxic-element processing is provided by studies showing that *ECS*- and *PCS*-deficient mutants of Arabidopsis are cadmium hypersensitive (Howden and Cobbett, 1992; Howden et al., 1995a, 1995b). In addition, Indian mustard (*Brassica juncea*) expressing transgenic ECS from a constitutive viral promoter show small increases in EC, GSH, and PC peptide levels. These plants are more cadmium resistant than the wild type (Zhu et al., 1999b) and accumulate 40% to 90% more cadmium in their shoots. Furthermore, transgenic Arabidopsis expressing very high levels of bacterial ECS and PCS have severalfold higher concentrations of EC, GSH, and PCs than the wild type, and show tolerance to arsenic and mercury (Dhankher et al., 2002; Li et al., 2004, 2005). In addition, the root-specific expression of PCS complemented the cadmium sensitivity of a *PCS*-deficient Arabidopsis mutant (Gong et al., 2003).

The following study examined the long-distance movement of EC-containing peptides from shoots to roots. The bacterial *ECS* gene was strongly expressed in shoots. The Arabidopsis ECS-deficient mutant, *cad2-1*, served as an excellent host for these experiments because it contains minimal levels of the thiol-peptide products from this pathway. Our results demonstrate unambiguously that EC was very efficiently transported from shoots to roots. Results on the tolerance to and accumulation of toxic elements by these complemented mutant plants are also presented.

RESULTS

Shoot-Specific Expression of the Bacterial ECS in Arabidopsis Mutant *cad2-1*

The Arabidopsis mutant *cad2-1* has dramatically reduced levels of ECS enzymatic activity due to a 6-base deletion within the sequence of the *ECS* gene (Cobbett et al., 1998). Homozygous mutant plants are hypersensitive to cadmium, mercury, and arsenic (Howden et al., 1995a; Ha et al., 1999). To examine the possible long-distance transport of the thiol-peptide products produced by ECS, the mutant *cad2-1* was engineered to specifically express the *Escherichia coli* ECS protein in shoot tissues under control of a light-induced, shoot-specific expression system with 5′ and 3′ regulatory sequences derived from the soybean (*Glycine max*) Rubisco small subunit *SRS1* gene, as shown in Figure 1, B and C. The *S1pt*::*ECS* gene was transformed into *ECS*-deficient *cad2-1* homozygous mutant Arabidopsis plants.

ECS protein expression levels were examined among T_2 generation *S1pt*::*ECS*-complemented *cad2-1*

mutant plant lines with western assays on 3-week-old plants using a monoclonal antibody, mAbECS1a, specific for the 57-kD bacterial ECS protein (Li et al., 2001). As shown in Figure 2, high levels of ECS protein were easily detected in shoots, but not roots, of the representative *S1pt*::*ECS*-expressing lines (e.g. CS1, CS4, and CS6) and not in wild-type controls. These results suggest that bacterial ECS protein was neither synthesized in roots nor transported from shoots to roots. Preliminary experiments demonstrated that these and other *S1pt*::*ECS* plant lines expressing high levels of ECS were similarly tolerant to arsenic. Furthermore, these three plant lines grew approximately as well as unchallenged wild-type control plants on Murashige and Skoog (MS) salt medium. Therefore, the CS1 line was chosen as representative and carried forward for a more detailed analysis.

Long-Distance Transport of Thiol-Peptides from Shoots to Roots in the *S1pt*::*ECS*-Complemented Mutant Plants Grown with or without Toxicants

We quantified thiol-peptide and Cys levels in the shoots and roots of the *cad2-1* mutant, the CS1 line, and wild-type plants to determine the effectiveness of expressing ECS in shoots and the ability of EC or other intermediates in the PC pathway to be transported to roots. Because thiol-peptide accumulation in plants can be greatly enhanced by elevated concentrations of toxic heavy metals and metalloids, 3-week-old plants grown in liquid culture were transferred to fresh media for 72 h with and without relatively low concentrations of arsenate, cadmium, and mercury (see "Materials and Methods"). Thiol-peptide compositions of shoots and roots from treated plants were compared to those from plants grown on normal medium alone. Monobromobimane (mBBr)-derivatized thiol-peptides from the leaves and roots of the various plants following different treatments were separated by HPLC and identified by their fluorescence, as shown for arsenic-treated samples in Figure 3.

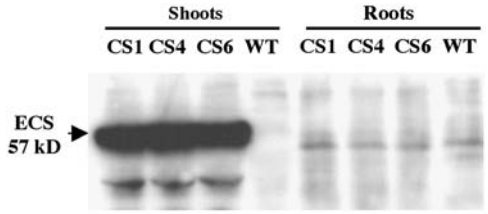

Figure 2. Western-blot analysis of ECS protein levels. ECS protein expression levels were examined in leaves and roots of 3-week-old plant lines *S1*::*ECS/cad2-1* using western blotting. The position of the 57-kD ECS protein band reacting with monoclonal antibody mAbECS1a is indicated. Protein samples (25 μg each) were from three lines where the *cad2-1* mutation is complemented with *S1pt*::*ECS* expression (CS1, CS4, and CS6) and wild type (WT). Western-blot assays were prepared as described previously for transgenic plants expressing ECS constitutively (Li et al., 2001, 2005).

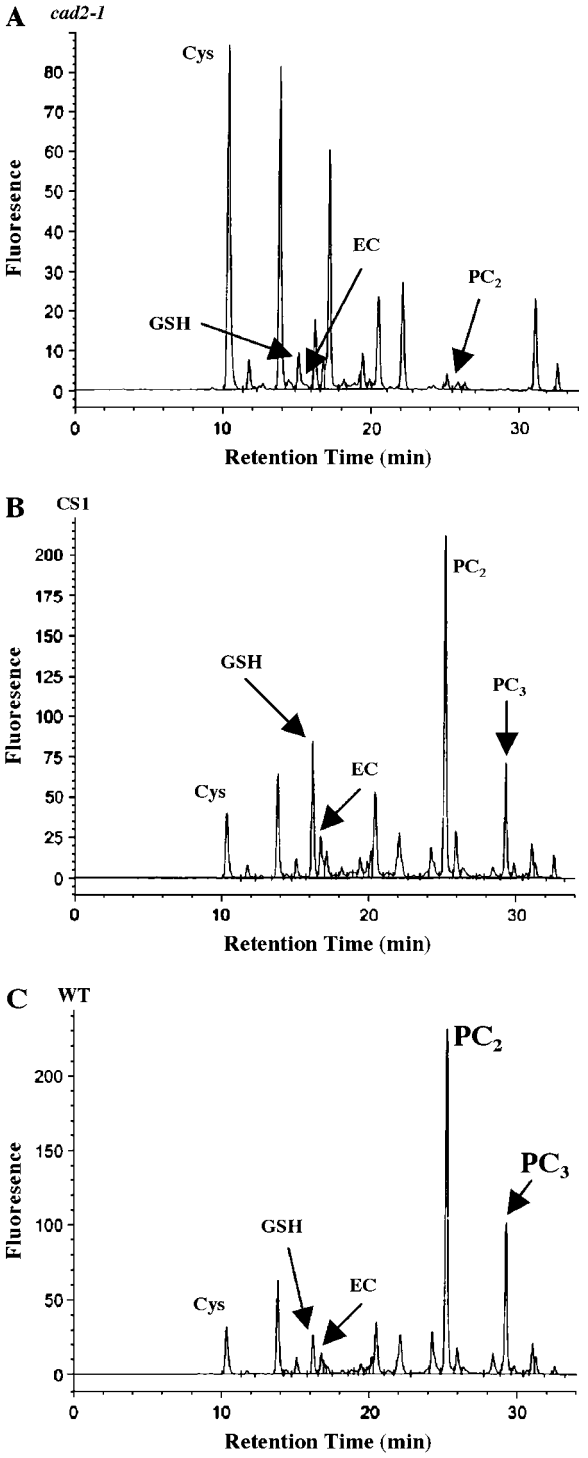

Figure 3. Fluorescence HPLC chromatograms of the mBBr-labeled thiol-peptides. A to C, The fluorescent mBBr-derivatized extracts from the roots of *cad2-1* (A), transgenic CS1 (B), and wild type (WT; C) Arabidopsis (var Columbia) plants after exposure to 100 μM arsenate for 72 h. Peak positions corresponding to Cys, EC, GSH, PC_2, and PC_3 are indicated as determined from the individual mBBr derivatives run as standards.

The levels of EC and GSH were relatively low in the shoots and roots of the *cad2-1* mutant plants, and PCs were barely detectable under all the treatments examined, as quantified in Figure 4. In contrast, significant 2- to 6-fold higher levels of EC were detected in both shoots and roots of the CS1 plants compared to the *cad2-1* mutant with or without exposure to toxicants (Figs. 3B and 4). Levels of EC in the roots of the CS1 line were equivalent to or higher than those in wild-type roots. GSH levels in the shoots and roots of CS1 plants were 7- to 40-fold greater than in the *cad2-1* mutant and 2- to 5-fold greater than in the wild type (Fig. 4C). The elevated levels of GSH in the transgenic line appeared to be relatively independent of treatments with toxic elements. Following arsenic treatment, increases were observed in the shoot and root levels of PC_2 and PC_3 in both the CS1 and wild-type plants relative to the *cad2-1* mutant. Surprisingly, the highest levels of PC_2 and PC_3 were found in the roots, but not shoots, of the CS1 plants, similar to the levels in the wild type (Fig. 4, D and E). These PCs were barely detected in the *cad2-1* mutant. These data suggest that there was sufficient EC transported to roots of CS1 plants for maximal arsenic-induced PC synthesis. Considering that PC levels were relatively low in the shoots of CS1 plants, it seems less likely that GSH and PC peptides were made in shoots and transported efficiently to roots. Exposure to cadmium produced small increases in PC_2 and PC_3 in the shoots and/or roots of both wild-type and CS1 plants (Fig. 4, D and E). There were no detectable PCs produced in response to mercury treatment in either wild-type or CS1 plants. To keep the sensitive *cad2-1* parental plants viable during these experiments, relatively low cadmium and mercury concentrations were used ("Materials and Methods"). It is possible that a transient exposure to higher cadmium or mercury concentrations would have produced greater increases in PC levels in the CS1 plants, as observed in wild-type plants and plants overexpressing ECS constitutively in all organs (Li et al., 2005).

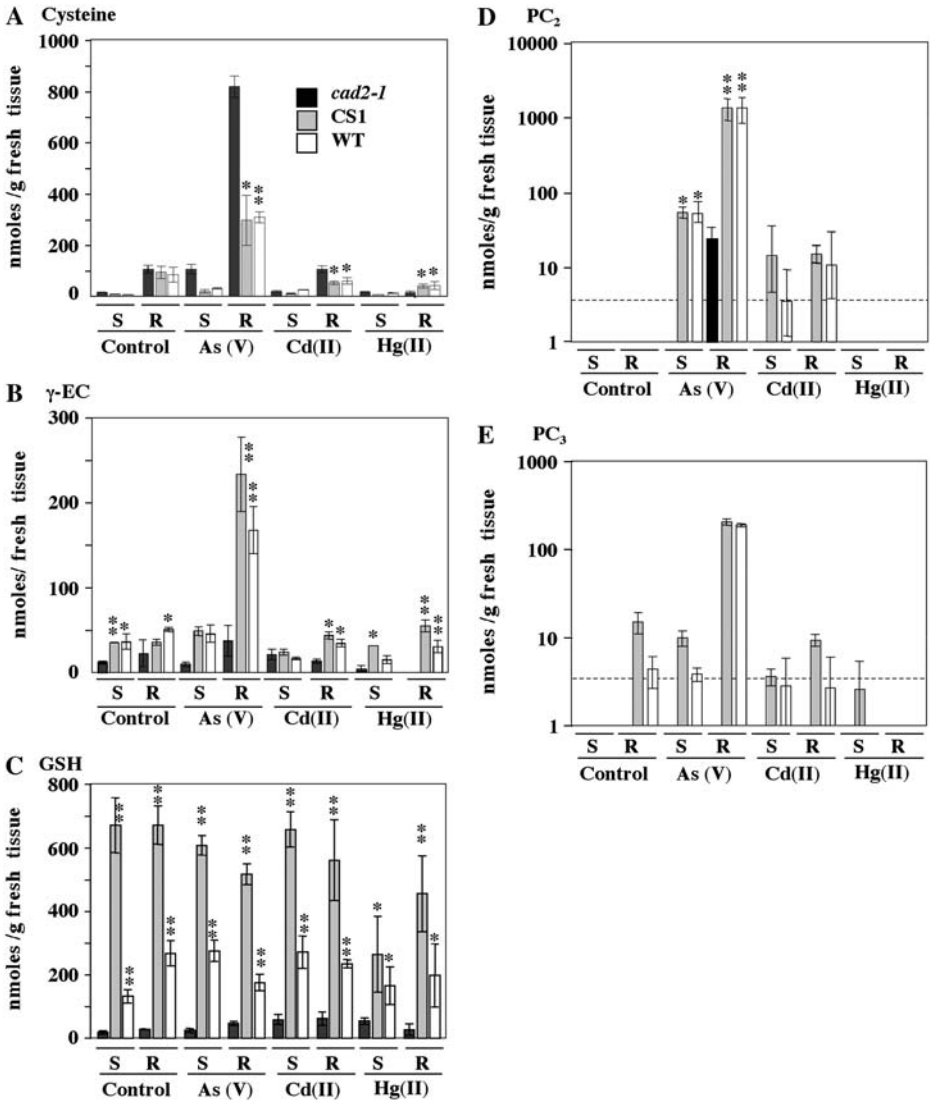

Figure 4. Comparison of Cys, EC, GSH, and PC levels in leaves and roots of wild-type, CS, and *cad2-1* plants. Transgenic line CS1 showed increased levels of EC, GSH, PC_2, and/or PC_3 peptides compared to mutant *cad2-1* plants when transferred to fresh media without (Control) or with 100 μM Na_3As0_4 [As(V)], 25 μM $CdCl_2$ [Cd(II)], or 5 μM $HgCl_2$ [Hg(II)] for 72 h. A to E, Levels of Cys, EC, GSH, PC_2, and PC_3 were normalized to the fresh weight of roots and shoots under different growth conditions, respectively, as indicated in the bar graphs. The data in D and E are plotted on a log scale to better illustrate the low levels of PC_2 and PC_3 in some samples. The limit of detection for a MBBr-derived peptide in any one experimental assay was approximately 3 nmol/g of fresh tissue as indicated by the dashed line. The data shown here were the average ± SEM of at least three independent experiments, and each experiment comprised 25 individual plants. Significant differences of the CS1 line and wild type from *cad2-1* are indicated by one ($P < 0.05$) or two asterisks ($P < 0.01$).

Cys is a substrate both for ribosomal synthesis of proteins and for the enzymatic synthesis of EC-containing peptides. Higher levels of Cys were observed in most *cad2-1* mutant plant samples (Fig. 4A) compared to wild-type or CS1 plants, as might be expected if ECS expression creates a significant drain on Cys. Furthermore, if ECS overexpression in the CS1 line created too great a demand on Cys pools, this might be expected to adversely affect protein synthesis and plant growth. However, CS1 plants grew equivalently to *cad2-1* mutants on media lacking toxicants. Arsenate treatment significantly increased the level of Cys in roots and shoots of *cad2-1* mutant, CS1, and wild-type plants compared to normal medium controls or treatment with cadmium or mercury (Fig. 4A). The shoot and root Cys levels in CS1 and wild-type plants were equivalent, even though all EC synthesis was occurring in the shoots of CS1 plants and most thiol-peptides were found at higher levels in the CS1 plants than the wild type. This result suggests that the root and shoot Cys levels in the CS1 plants were buffered by increased Cys synthesis and/or transport. Arsenic treatment caused the most significant increases in Cys levels of all plants relative to treatment with mercury or cadmium, suggesting it had a positive role in inducing the Cys biosynthetic pathway.

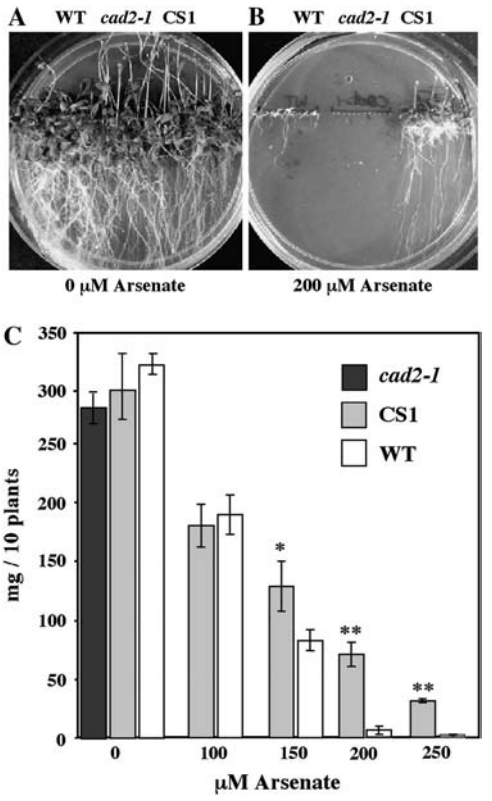

Figure 5. Arsenate resistance of the CS1 transgenic plants was compared to wild type. A and B, Sterilized seeds were plated onto half-strength MS phytagar as a control (A) or on half-strength MS supplemented with 200 μM arsenate (Na_3AsO_4; B). After germination, the plates were incubated in a vertical orientation for 5 weeks. C, The fresh weights of plants grown with various arsenate concentrations in the media were quantified after 3 weeks. Comparing each CS1 experimental to wild type, the data shown are the average ± SEM of three independent experiments, each experiment consisting of weighing 10 individual plants. Significant differences from wild type are indicated with one ($P < 0.05$) or two asterisks ($P < 0.002$).

Shoot-Specific Expression of ECS Suppressed the Arsenic, Mercury, and Cadmium Hypersensitivity of the *cad2-1* Mutant

Seed germination and plant growth in media without and with toxicants were compared among the mutant *cad2-1*, wild-type, and CS1 plants, using T_2 generation CS1 seeds. Seeds of all three genotypes germinated efficiently and all plants grew similarly on half-strength MS salts (Fig. 5A). The *S1pt::ECS* transgene in the CS1 plants suppressed the sensitivity phenotype of the mutant and resulted in significant levels of arsenate tolerance (Fig. 5B), although they did not grow nearly as well as unchallenged control plants (Fig. 5A). The CS1 plants were more resistant than wild type to arsenate concentrations ranging from 150 to 250 μM, as quantified in Figure 5C. The *cad2-1* mutant seeds did not germinate on media containing 100 μM to 250 μM arsenate; therefore, no quantitative data for growth of the mutant on arsenic are shown.

All three genotypes germinated on 30 μM $HgCl_2$, but this concentration completely inhibited growth of the *cad2-1* plants (Fig. 6). The transgenic CS1 seeds germinated, and plants grew in media containing 30 to 50 μM $HgCl_2$ and showed mercury tolerance similar to wild-type plants. Thus, the mercury hypersensitivity of the *cad2-1* mutant was suppressed and wild-type levels of tolerance were restored by the shoot-specific expression of the *S1pt::ECS* gene (Fig. 6, B and C).

The *cad2-1* allele was originally isolated based on a cadmium-hypersensitive plant phenotype (Howden and Cobbett, 1992). The *cad2-1*, CS1, and wild-type plants were able to germinate on media containing 50 and 75 μM $CdCl_2$, but not on higher concentrations of the toxicant shown in Figure 7. Based on fresh shoot weight, the CS1 transgenic plants consistently showed significant Cd(II) tolerance relative to the *cad2-1* mutant on media with 75 to 100 μM cadmium (Fig. 7, B and C). However, the CS1 plants grew slowly and showed more sensitivity to Cd(II) than wild type. The sensitivity of the CS1 and *cad2-1* plants compared to the wild-type plants was statistically significant following 75 and 100 μM cadmium treatments ($P < 0.0005$–0.05).

Element Accumulation in Shoots

It seemed reasonable to expect that EC, GSH, and PCs would complex with thiol-reactive elements and result in increased accumulation of these toxicants in aboveground tissues. In other words, thiol-peptides

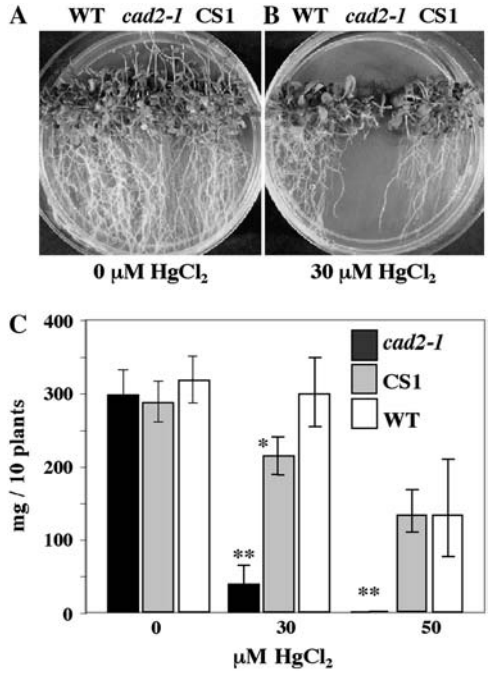

Figure 6. Mercury resistance assay. A and B, Wild-type, CS1, and *cad2-1* plants grown without heavy metals (A) or with 30 μM Hg(II) (B) for 5 weeks. C, The fresh weights of plants grown for 3 weeks without or with 30 or 50 μM $HgCl_2$were determined. The data shown are the average ± SEM of three independent experiments, each experiment consisted of weighing 10 individual plants. Significant differences from wild type are indicated by one ($P < 0.05$) or two asterisks ($P < 0.002$).

provide a chemical sink for these reactive elements, and perhaps the thiol-peptide-element complexes would be stored at high levels in vacuoles as they are in yeast (*Saccharomyces cerevisiae*; Li et al., 1997; Sharma et al., 2002). To test for element accumulation, *cad2-1* mutant, wild-type, and CS1 seeds were germinated in media with 25, 50, and 150 μM arsenate; 15 and 30 μM $CdCl_2$; or 15 and 30 μM $HgCl_2$. Plants were grown for 3 weeks, and the arsenic, cadmium, and mercury concentrations were quantified in the shoot tissues of these plants using inductively coupled plasma-optical mass spectroscopy, as shown in Figure 8. Growing in media containing 25 μM arsenate for 3 weeks, the mutant *cad2-1* accumulated 2-fold more arsenic in aboveground tissues than did wild-type or CS1 plants. Only minor differences in accumulation were found between wild-type and CS1 plants on higher concentrations (Fig. 8A). Growing on media with 15 and 30 μM $HgCl_2$ for 3 weeks, the CS1 plants accumulated significantly more mercury in aboveground tissues than wild-type or mutant *cad2-1* plants (Fig. 8B). When the plants were exposed to 15 μM Cd(II), there were no differences in cadmium accumulation among the *cad2-1* mutant, the CS1, or wild-type plants (Fig. 8C). Similarly, there was no difference between the CS1 or wild-type plants on 30 μM $CdCl_2$.

DISCUSSION

Long-Distance Shoot-to-Root Transport of the Thiol-Peptides and Bound Elements

This study examined the long-distance transport of the thiol-peptide EC from shoots, where it was synthesized, to roots. Recent publications have begun to identify the molecular genetic basis for systems controlling the long-distance transport of amino acids and nitrogen (Hirner et al., 1998; Fischer et al., 2002), purines (Burkle et al., 2003), sodium (Shi et al., 2002), and phosphate (Hamburger et al., 2002; Wang et al., 2004). EC, GSH, and PCs are rich sources of both nitrogen and sulfur, and may be efficiently transported for the redistribution of these nutrients and to supply them as substrates in various stress responses. The *S1pt::ECS* transgene, encoding the first enzymatic step in the PC biosynthetic pathway (Fig. 1), was specifically expressed in the shoots of the Arabidopsis ECS-deficient *cad2-1* mutant. The *S1pt::ECS* plants expressed the bacterial ECS protein at high levels in the shoots, but not in roots, demonstrating the organ-specific, aboveground ECS expression from the transgene and, as expected, no phloem movement of this relatively large 57-kD protein (Fig. 2). The roots of the

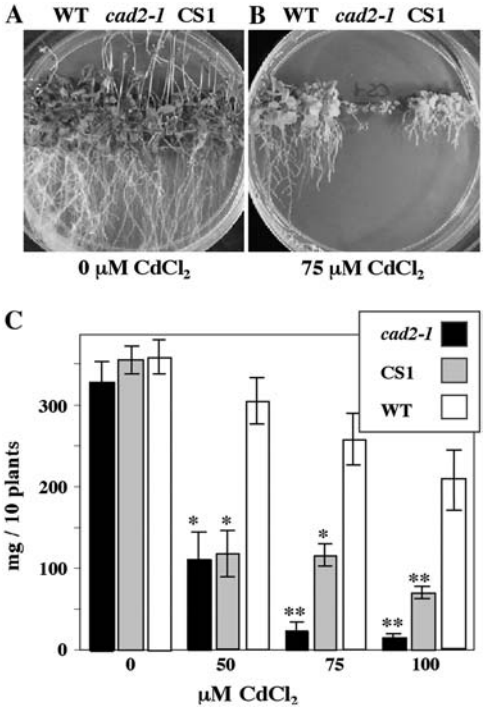

Figure 7. Cadmium sensitivity of the transgenic plants CS1 compared to wild type. A and B, Sterilized seeds were plated onto half-strength MS phytagar as a control (A) or on half-strength MS supplemented with 75 μM cadmium ($CdCl_2$; B). After germination, the plates were incubated in a vertical orientation for 5 weeks. C, The fresh weights of plants grown under the various cadmium stress conditions were quantified after 3 weeks of growth. Significant differences from wild type are indicated by one ($P < 0.05$) or two asterisks ($P < 0.01$).

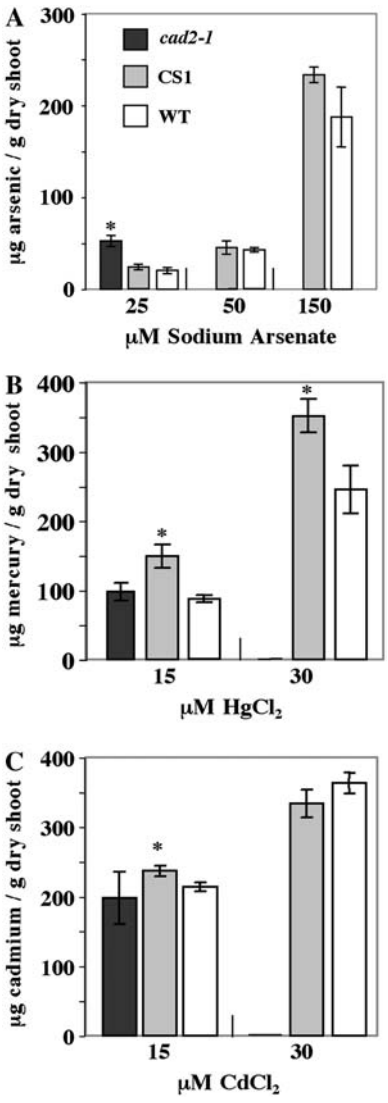

Figure 8. Arsenic, mercury, and cadmium accumulation in aboveground tissues of CS1, *cad2-1*, and wild-type plants. The shoot tissues were collected from the seedlings grown in half-strength MS phytagar medium supplied with 25, 50, and 150 μM arsenate (A); 10 and 30 μM $HgCl_2$ (B); or 15 and 30 μM $CdCl_2$ (C) for 3 weeks. Comparing each experimental to wild type, the data shown are the average ± SEM of three independent experiments and are normalized to micrograms of metal or metalloid per gram dry plant tissue. Significant differences from wild type are indicated with an asterisk ($P < 0.05$).

cad2-1 mutant had extremely low levels of EC, GSH, and PCs, establishing a low background in which to examine ECS enzyme expression. The majority of the molecular peptide phenotypes of the *cad2-1* mutant were complemented by expression of the *S1pt::ECS* transgene. For example, the roots of the *S1pt::ECS* transgenic plants had much higher levels of EC and GSH than the mutant, levels that were as high or higher than wild type under all growth conditions. After 48 h of treatment with arsenic, the root, but not shoot, levels of PC_2 and PC_3 were also enhanced to wild-type levels. These results suggest that shoot-specific expression of ECS was sufficient to supply EC and GSH thiol-peptides to shoots and roots.

The levels of GSH were enhanced in shoots and roots dramatically above wild-type levels, with or without treatment with toxic elements. GSH levels were enhanced to similarly high levels in shoots and roots, suggesting GSH is more efficiently transported than EC from shoots to roots. However, EC peptide levels were significantly elevated in roots of the CS1 transgenic plants, making it possible that GSH synthesis was also enhanced in the roots of these plants. It should be noted that GSH synthesis and/or transport in the CS1 plants does not appear to be dependent upon other stress signals or other systems of induced phloem transport. Furthermore, the elevated GSH levels in transgenic plants suggest that EC substrate concentrations are all that limited GS-catalyzed synthesis of GSH (Fig. 1) in both the wild type and *cad2-1* mutants, as suggested by previous studies in diverse prokaryotes and eukaryotes (Wu and Moye-Rowley, 1994; Grondin et al., 1997; May et al., 1998; Manna et al., 1999).

The fact that arsenic could more efficiently induce the biosynthesis of PCs in roots of these plants is not unexpected once the roots contain high levels of EC and GSH. Enhanced root PC synthesis may have occurred via the direct activation of PCS enzyme activity by arsenic (Fig. 1; Vatamaniuk et al., 1999, 2000). However, it is hard to explain why root PC levels were not similarly enhanced after cadmium treatment because Cd(II) is known to efficiently enhance PCS activities. Arsenic might have increased the stability of PC peptides more efficiently than Cd(II) and Hg(II). Withdrawing arsenic and cadmium for set time periods would allow an examination of the decay profiles of PC complexes. The possibility that arsenic more efficiently enhanced the shoot-to-root transport of these peptides than the mercury or cadmium treatments seems unlikely, because the arsenic-treated plants do not have more highly elevated GSH levels than the mercury- or cadmium-treated plants.

A previous study by Gong et al. (2003) used the root-specific expression of PCS to demonstrate the apparent xylem transport of thiol-peptides up to leaves. Our results using the leaf-specific expression of ECS demonstrate the existence of a complementary activity—EC can be synthesized in the shoots, and EC and apparently GSH are transported via the phloem down to roots. The long-distance phloem transport of EC and GSH has potential implications for the cotransport of bound thiol-reactive nutrients and toxic elements. Phloem transport of these thiol-peptides and their element complexes could provide an important mechanism for the lateral redistribution of nutrients like copper and zinc brought up from roots or the return of these nutrients to roots during leaf senescence in perennial plants. Several essential plant nutrient metal ions, including Zn(II), Cu(II), and Co(II), have reasonably strong affinities for Cys thiols. It has recently been demonstrated that a Zn-GSH or Zn-(GS)2 peptide

complex acts as both the peptide donor and acceptor in the PCS-catalyzed formation of PCs (Vatamaniuk et al., 2000). However, our preliminary efforts to demonstrate the downward movement of arsenic applied to the shoots to the roots of CS1 plants did not detect any significant levels of root arsenic (data not shown). Future research will be needed to determine the precise roles of EC-containing peptides in conutrient or cotoxicant transport (Meagher and Heaton, 2005; Meagher et al., 2006). Finally, because phloem transport must involve many plasma membrane-to-plasma membrane intracellular transporters, our data suggests there is a great deal to learn about the molecular genetic basis for the rapid shoot-to-root movement of thiol-peptides in plants.

The Shoot-Specific Expression of Bacterial ECS Complemented the Arsenic-, Mercury-, and Cadmium-Sensitive Phenotypes of the *cad2-1* Mutant

The *cad2-1* mutant is hypersensitive to arsenic, mercury, and cadmium due to a block in EC synthesis and, hence, the PC pathway (Fig. 1). EC, GSH, and PCs play critical roles in toxic-element detoxification and stress responses (Cobbett and Goldsbrough, 2002; Cobbett and Meagher, 2003). With high levels of shoot-specific expression of the bacterial ECS, the *S1pt::ECS* plants showed significant tolerance to arsenic, mercury, and cadmium compared to *cad2-1* plants. Considering the toxicity of these elements, it is unlikely that root tissues would have remained alive and functional in the *S1pt::ECS*-complemented *cad2-1* lines had it not been for the efficient phloem transport of EC and/or GSH to roots.

The increased arsenate resistance associated with ECS expression in a *cad2-1* mutant background (e.g. the CS1 line) was most striking. In fact, arsenate resistance in CS1 exceeded that observed for the wild-type plants. The oxyanion arsenate (AsO_4^{-3}) is a chemical analog of phosphate (PO_4^{-3}). There is reasonable evidence that arsenate is erroneously pumped into both yeast and plant cells by phosphate transporters (Ullrich-Eberius et al., 1989; Bun-ya et al., 1996), most likely due to the structural similarity of the two compounds (Ullrich-Eberius et al., 1989; Bun-ya et al., 1996). Arabidopsis plants deficient in root phosphate transporters are moderately resistant to arsenate (Shin et al., 2004). Arsenate is efficiently reduced into arsenite (AsO_3^{-3}) by GSH-dependent arsenate reductase activities in bacteria, fungi, plants, and animals (Oden et al., 1994; Mukhopadhyay et al., 1998; Dhankher et al., 2002, 2006; Dong et al., 2005). Arsenite is highly thiol-reactive and may be chelated and sequestrated as $As(S\text{-}R)_3$ thiol-peptide complexes during its detoxification (Dey et al., 1994; Rosen, 1999; Sharples et al., 2000). Thus, the significant levels of arsenic tolerance observed for the *S1pt::ECS/cad2-1* transgenic CS1 plants examined in this study must be at least in part due to high levels of EC and its downstream products in both roots and shoots relative to wild-type plants. The GSH levels surpassed those in wild type, and this may account for the greater resistance of the CS1 line to arsenic than wild type. Finally, these abnormally high levels of GSH may enhance GSH-dependent oxidative stress pathways, above the levels that can commonly be induced by toxins, thus providing increased resistance (Xiang et al., 2001; Ball et al., 2004).

The CS1 plants showed significant tolerance to mercury and cadmium compared to the *cad2-1* mutant plants. The leaf-specific expression of the *S1pt::ECS* transgene restored mercury resistance to near wild-type levels (Fig. 6). Hg(II) is highly thiol-reactive and forms extremely strong bonds with sulfur, but there are many other chemical activities resulting in mercury toxicity (Rugh et al., 1996; Bizily et al., 2003). In response to Hg(II) stress, the levels of EC and GSH in the roots and shoots of CS1 plants were higher than in wild-type plants, whereas mercury caused no significant increases in the levels of PCs. These results suggest that the levels of EC and GSH were essential to mercury resistance, but the levels of these peptides alone were not directly proportional to mercury resistance or the transgenic plants would have been more mercury resistant than wild type. Perhaps the GSH-dependent oxidative stress-response pathway indirectly protects cells from the toxic effects of mercury, and this pathway is not enhanced when GSH levels are increased beyond wild-type levels. Furthermore, the lack of observed increases in PCs suggests that mercury may not enhance the enzymatic activity of PCS as effectively as other thiol-reactive metal ions like Cd(II) (Vatamaniuk et al., 2000, 2004). Although, transgenic PCS expression did enhance the resistance of yeast to arsenic, mercury, and cadmium (Vatamaniuk et al., 1999). Consistent with the view that PCS is not activated by mercury are our data showing that the strong overexpression of Arabidopsis PCS only weakly increases mercury resistance in transgenic Arabidopsis (Li et al., 2004). In light of the CS1 line's resistance to arsenic and mercury, it might seem surprising these plants were relatively poorly complemented for Cd(II) resistance relative to the levels of resistance for wild type (Fig. 7). However, even wild-type Arabidopsis that are strongly and constitutively overexpressing either ECS or PCS in shoots and roots are hypersensitive to cadmium, suggesting that Arabidopsis may have special problems processing the cadmium-thiol-peptide complexes (Li et al., 2004, 2005).

Role of EC-Containing Peptides in Accumulation of Toxicants

The *S1pt::ECS* complemented Arabidopsis *cad2-1* mutants in the expression of high levels of several thiol-peptides aboveground, but did not accumulate more arsenic or cadmium aboveground compared to the *cad2-1* mutant or wild-type plants and only accumulated about 30% more mercury. One possible explanation for these results is that toxicants taken up by roots were chelated to form the thiol-peptide-metal

complexes, which themselves do not move to the aboveground tissues; only the toxicants in the form of free ions or in complexes with other non-thiol-peptide carriers like His may be transported to the shoots. By this proposal, the amount of the shoot-transportable toxicant available would be inversely proportional to the levels of thiol-peptides in roots. This inverse relationship is supported by the fact that, at a very low arsenic concentration where the *cad2-1* mutant grew and had extremely low levels of these thiol-peptides, it accumulated 2-fold more arsenic aboveground than the CS1 line or the wild type (Figs. 4 and 8). Similar results were reported for the *cad2-1* mutant relative to wild type (Howden et al., 1995a; Cobbett et al., 1998). Contrary to the view of thiol-peptides sequestering the toxicants in roots are the recent data of Gong et al. (2003). Using the root-specific expression of Arabidopsis PCS to enhance PC levels, they demonstrate the apparent xylem transport of thiol-peptide metal-ion complexes up to leaves, and increased accumulation of cadmium over what occurs in the *cad1-3 PCS* mutant. Our preliminary attempts to show altered rates of arsenic movement from shoots to roots of the CS1 line did not reveal any differences relative to wild type or the *cad2-1* mutant (data not shown).

The levels of EC peptide in the transgenic CS1 plants were 2- to 10-fold greater than in *cad2-1* plants under various growth conditions. The CS1 plant levels of GSH were 2- to 10-fold higher than in the mutant and 2- to 5-fold higher than wild type with or without mercury treatment. And yet, the level of mercury accumulation in the aboveground tissues of the CS1 plants was only 65% higher than in wild-type plants (Fig. 8B). As with arsenic and cadmium, there is little correlation between mercury accumulation and the thiol-peptide content of these plants.

CONCLUSION

Shoot-specific expression of a bacterial *ECS* in the Arabidopsis *cad2-1* mutant significantly increased levels of EC and GSH in both roots and shoots of the transgenic Arabidopsis plants compared to mutant plants, and GSH levels in roots were increased beyond those in wild type. These data demonstrate the long-distance phloem transport of the EC peptide and perhaps other thiol-peptides from shoots to roots. Considering the high sulfur and nitrogen content of these peptides, phloem transport may be essential for the proper distribution of these nutrients. The shoot-specific expression of ECS complemented mutant sensitivities to three thiol-reactive toxicants: the *S1pt::ECS* plants were relatively tolerant to arsenate, mercury, and cadmium compared to the mutant itself, and even more tolerant to arsenate than wild type. Increasing tolerance is one of the most important criteria in designing plants that can be used in various phytoremediation schemes. However, there is no simple direct relationship between increasing levels of thiol-peptides and increases in aboveground accumulation of thiol-reactive toxic elements.

MATERIALS AND METHODS

Cloning and Shoot Tissue-Specific Expression of Bacterial ECS

The coding sequence of the *Escherichia coli* SK1592 *ECS* gene was cloned previously by Li et al. (2001). A light-induced, leaf-specific expression vector with the promoter region and terminator from *SRS1* (termed collectively *S1pt*) was used to express the *ECS* gene in plants (Fig. 1A). The *SRS1* promoter region was previously described for the *S1p* vector by Dhankher et al. (2002). The *S1pt* cassette was constructed by adding the terminal 3′-untranslated region (UTR) and polyadenylation signals to *S1p* (Shirley et al., 1987, 1990; Fig. 1B). The ECS coding sequence was cloned into the *Nco*I/*Hin*dIII replacement region of the *S1pt* cassette in pBluescript SK(II), and then the assembled construct was moved as a *Sac*I/*Xho*I fragment into the replacement region of the binary vector pBin19 to make *S1pt::ECS*. The above construct was introduced into the *cad2-1* mutant of Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia) by Agrobacterium-mediated transformation according to Ye et al. (1999). The sterilized T_1 transgenic seeds were plated onto MS medium (Murashige and Skoog, 1962) and supplemented with kanamycin (50 μg/mL) and timentin (300 μg/mL) for 3 weeks before transplanting the surviving seedlings into soils.

ECS expression was compared among transgenic, mutant, and control plants on western blots (Li et al., 2001) using an *E. coli* ECS-specific monoclonal antibody as described previously (Li et al., 2001). Primary antibody was followed by horseradish peroxidase-conjugated goat antimouse or antirabbit antisera, respectively (Sigma), and enhancement using an ECL kit from Amersham following the manufacturer's instructions. Protein concentrations of the plant extracts were determined by a Bradford assay (Bradford, 1976). Equal loading of each sample was further confirmed by Coomassie Brilliant Blue G staining of aliquots run previously on separate gels (data not shown). The concentration of ECS in shoots was similar to that determined for the *ECS* gene expressed from the constitutive actin *ACT2* promoter (Li et al., 2005), or approximately 0.1% of total shoot protein (data not shown).

Plant Growth, and Heavy-Metal and Metalloid Treatments

The Arabidopsis wild type, its mutant *cad2-1*, and the transgenic *S1::ECS/cad2-1* plants were grown for all assays with cycles of 16 h light and 8 h darkness at 22°C (±1°C) on agar or in liquid media (Li et al., 2005). To test metal and metalloid resistance, the sterilized seeds (Li et al., 2001) were plated onto solid 0.8% plant tissue culture grade agar (Caisson Laboratories) containing half-strength MS (Murashige and Skoog, 1962) medium and various levels of arsenate (Na_3AsO_4), mercury ($HgCl_2$), or cadmium ($CdCl_2$; Figs. 5–7). After the seeds germinated, the plates were vertically positioned and grown for 3 weeks before the shoot fresh weight was quantified. Three sets of 10 plants were weighed for each plant genotype and treatment. To determine thiol-peptide levels, 3-week-old plants that were grown on platforms in liquid MS media and were prepared for HPLC assays after 72-h exposure to 25 μM $CdCl_2$ or 100 μM Na_3AsO_4 as described by Li et al. (2005). The Cd(II)-treated *cad2-1* mutant roots turned brown, but the tissues and cells appeared intact. A lower concentration of 5 μM $HgCl_2$ was used instead of the 25 μM concentration used in previous studies (Li et al., 2004, 2005). In initial experiments, exposing *cad2-1* plants to 25 μM Hg(II) in liquid for 48 to 72 h caused the plant roots to turn black, lose cellular integrity, and lose more than 50% of their weight. Exposing *cad2-1* plants to 5 μM Hg(II) resulted in root tissues turning gray, suggesting they were still taking in mercury, but these tissues appeared intact and viable during the treatment.

HPLC Analysis of Thiol-Peptides

Cys and thiol-containing peptides EC, GSH, PC_2, and PC_3 were analyzed using fluorescence-detection HPLC as described (Fahey and Newton, 1987). Peptides were extracted and derivatized with mBBr as described previously (Sneller et al., 2000; Cazale and Clemens, 2001; Sauge-Merle et al., 2003) with

minor modifications (Li et al., 2005). In vitro-labeled Cys and peptides were prepared as described by Li et al. (2004).

Quantification of Arsenic, Mercury, and Cadmium in Shoot Tissues

Assays on the accumulation of arsenic, cadmium, or mercury in shoot tissues were conducted in liquid culture. The mutant *cad2-1* or transgenic *S1::ECS/cad2-1* plants were grown for 3 weeks in half-strength solid MS medium containing various low concentrations of arsenate (25, 50, and 150 μM), or Hg(II) (10 and 30 μM $HgCl_2$), or Cd(II) (15 and 30 μM $CdCl_2$). Shoots were harvested, and washed three to four times with deionized water to remove any traces of surface contamination. For mercury assay, the shoot tissues were lyophilized at −34°C for more than 72 h. . For arsenic or cadmium assays, the shoot tissues were dried at 80°C incubator instead of lyophilized. Shoot samples were ground to a powder prior to acid digestion, and elemental analysis was preformed as described by Suszcynsky and Shann (1995) and modified by Li et al. (2004).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers 08X900 and P00865.

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